

FORM PTO 1590 (REV 5-93) US DEPARTMENT OF COMMERCE PATENT AND TRADEMARK OFFICE		ATTORNEY DOCKET NUMBER 2002_0317A
TRANSMITTAL LETTER TO THE UNITED STATES DESIGNATED/ELECTED OFFICE (DO/EO/US) CONCERNING A FILING UNDER 35 U.S.C. §371		U.S. APPLICATION NO. (if known - 37 CFR 1.5) NEW <b>107070387</b>
International Application No. PCT/JP00/06103	International Filing Date September 7, 2000	Priority Date Claimed September 7, 1999
<b>Title of Invention</b> CYCLIC DEPSIPEPTIDE SYNTHETASE AND GENE THEREOF, AND MASS PRODUCTION SYSTEM FOR CYCLIC DEPSIPEPTIDE		
<b>Applicant(s) For DO/EO/US</b> Naoki MIDOH, Kaoru OKAKURA, Koichi MIYAMOTO, Manabu WATANABE, Koji YANAI, Tetsuya YASUTAKE, Sato AIHARA, Takafumi FUTAMURA, Horst KLEINKAUF, Takeshi MURAKAMI		
Applicant herewith submits to the United States Designated/Elected Office (DO/EO/US) the following items and other information:		
<ol style="list-style-type: none"> <li>1. <input checked="" type="checkbox"/> This is a <b>FIRST</b> submission of items concerning a filing under 35 U.S.C. §371.</li> <li>2. <input type="checkbox"/> This is a <b>SECOND</b> or <b>SUBSEQUENT</b> submission of items concerning a filing under 35 U.S.C. §371.</li> <li>3. <input type="checkbox"/> This express request to begin national examination procedures (35 U.S.C. §371(f)) at any time rather than delay examination until the expiration of the applicable time limit set in 35 U.S.C. §371(b) and PCT Articles 22 and 39(1).</li> <li>4. <input checked="" type="checkbox"/> A proper Demand for International Preliminary Examination was made by the 19th month from the earliest claimed priority date.</li> <li>5. <input checked="" type="checkbox"/> A copy of the International Application as filed (35 U.S.C. §371(c)(2)) <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> is transmitted herewith (required only if not transmitted by the International Bureau). <b>ATTACHMENT A</b></li> <li>b. <input checked="" type="checkbox"/> has been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> is not required, as the application was filed in the United States Receiving Office (RO/US)</li> </ul> </li> <li>6. <input checked="" type="checkbox"/> A translation of the International Application into English (35 U.S.C. §371(c)(2)). <b>ATTACHMENT A</b></li> <li>7. <input type="checkbox"/> Amendments to the claims of the International Application under PCT Article 19 (35 U.S.C. §371(c)(3)). <ul style="list-style-type: none"> <li>a. <input type="checkbox"/> are transmitted herewith (required only if not transmitted by the International Bureau).</li> <li>b. <input type="checkbox"/> have been transmitted by the International Bureau.</li> <li>c. <input type="checkbox"/> have not been made; however, the time limit for making such amendments has NOT expired.</li> <li>d. <input type="checkbox"/> have not been made and will not be made.</li> </ul> </li> <li>8. <input type="checkbox"/> A translation of the amendments to the claims under PCT Article 19.</li> <li>9. <input checked="" type="checkbox"/> An executed oath or declaration of the inventor(s) (35 U.S.C. §371(c)(4)). <b>ATTACHMENT B</b></li> <li>10. <input type="checkbox"/> A translation of the annexes to the International Preliminary Examination Report under PCT Article 36 (35 U.S.C. §371(c)(5)).</li> </ol>		
<b>Items 11. to 14. below concern other document(s) or information included:</b>		
<ol style="list-style-type: none"> <li>11. <input checked="" type="checkbox"/> An Information Disclosure Statement under 37 CFR 1.97 and 1.98. <b>ATTACHMENT C</b></li> <li>12. <input checked="" type="checkbox"/> An assignment document for recording. A separate cover sheet in compliance with 37 CFR 3.28 and 3.31 is included. <b>ATTACHMENT D</b></li> <li>13. <input checked="" type="checkbox"/> A FIRST preliminary amendment. <b>ATTACHMENT E</b> <input type="checkbox"/> A SECOND or SUBSEQUENT preliminary amendment.</li> <li>14. <input checked="" type="checkbox"/> Other items or information: Cover Letter with 3 Deposit Receipts - <b>ATTACHMENT F</b> and diskette containing Sequence Listing</li> </ol>		

THE COMMISSIONER IS AUTHORIZED  
TO CHARGE ANY DEFICIENCY IN THE  
FEE FOR THIS PAPER TO DEPOSIT  
ACCOUNT NO. 23-0975.

U.S. APPLICATION NO. <b>107070387</b>		INTERNATIONAL APPLICATION NO. PCT/JP00/06103	ATTORNEY'S DOCKET NO. 2002 0317A
15. [X] The following fees are submitted		<b>CALCULATIONS</b>	
<b>BASIC NATIONAL FEE (37 CFR 1.492(a)(1)-(5)):</b>			
Neither international preliminary examination fee nor international search fee paid to USPTO and International Search Report not prepared by the EPO or JPO . . . . . \$1040.00			
International Search Report has been prepared by the EPO or JPO . . . . . \$ 890.00			
International preliminary examination fee not paid to USPTO but international search paid to USPTO . . . . . \$ 740.00			
International preliminary examination fee paid to USPTO but claims did not satisfy provisions of PCT Article 33(1)-(4) . . . . . \$ 690.00			
International preliminary examination fee paid to USPTO and all claims satisfied provisions of PCT Article 33(1)-(4) . . . . . \$ 100.00			
<b>ENTER APPROPRIATE BASIC FEE AMOUNT =</b>		\$890.00	
Surcharge of \$130.00 for furnishing the oath or declaration later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(e)).		\$	
<b>Claims</b>		<b>Number Filed</b>	<b>Number Extra</b>
Total Claims	22 -20 =	2	X \$18.00      \$36.00
Independent Claims	- 3 =		X \$84.00      \$
Multiple dependent claim(s) (if applicable)			+ \$280.00      \$280.00
<b>TOTAL OF ABOVE CALCULATIONS =</b>		\$1,206.00	
[ ] Small Entity Status is hereby asserted. Above fees are reduced by 1/2.		\$	
<b>SUBTOTAL =</b>		\$1,206.00	
Processing fee of \$130.00 for furnishing the English translation later than [] 20 [] 30 months from the earliest claimed priority date (37 CFR 1.492(f)).		+ \$	
<b>TOTAL NATIONAL FEE =</b>		\$1,206.00	
Fee for recording the enclosed assignment (37 CFR 1.21(h)). The assignment must be accompanied by an appropriate cover sheet (37 CFR 3.28, 3.31). \$40 per property +		\$40.00	
<b>TOTAL FEES ENCLOSED =</b>		\$1,246.00	
		Amount to be refunded      \$	
		Amount to be charged      \$	
<p>a. [X] A check in the amount of <u>\$1,246.00</u> to cover the above fees is enclosed. A duplicate copy of this form is enclosed.</p> <p>b. [ ] Please charge my Deposit Account No. 23-0975 in the amount of \$ _____ to cover the above fees. A duplicate copy of this sheet is enclosed.</p> <p>c. [ ] The Commissioner is hereby authorized to charge any additional fees which may be required, or credit any overpayment to Deposit Account No. <u>23-0975</u>.</p>			
<p><b>NOTE: Where an appropriate time limit under 37 CFR 1.494 or 1.495 has not been met, a petition to revive (37 CFR 1.137(a) or (b)) must be filed and granted to restore the application to pending status.</b></p>			
19. CORRESPONDENCE ADDRESS		<p>By: <u>Warren M. Cheek</u>            Warren M. Cheek, Jr.,            Registration No. 33,367</p> <p>WENDEROTH, LIND &amp; PONACK, L.L.P.            2033 "K" Street, N.W., Suite 800            Washington, D.C. 20006-1021            Phone: (202) 721-8200            Fax: (202) 721-8250</p>	
 <b>000513</b> PATENT TRADEMARK OFFICE		<p>March 6, 2002</p>	

**19. CORRESPONDENCE ADDRESS**



000513

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March 6, 2002

[CHECK NO.]

[2002\_0317A]

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re application of

Naoki MIDOH et al. : Attn: BOX PCT

Serial No. NEW : Docket No. 2002\_0317A

Filed March 6, 2002

CYCLIC DEPSIPEPTIDE SYNTHETASE AND  
GENE THEREOF, AND MASS PRODUCTION  
SYSTEM FOR CYCLIC DEPSIPEPTIDE  
[Corresponding to PCT/JP00/06103  
Filed September 7, 2000]

PRELIMINARY AMENDMENT

Assistant Commissioner for Patents,  
Washington, DC 20231

Sir:

Prior to calculating the filing fee, please amend the above-identified application as follows:

IN THE SPECIFICATION

**Page 1, immediately after the title, please insert:**

This application is a 371 of PCT/JP00/06103 filed September 7, 2000.

IN THE CLAIMS

**Please amend the claims as follows:**

**7. (Amended) A recombinant vector comprising the polynucleotide of claim 2 or claim 4.**

**10. (Amended) The host according to claim 8, which is a substance PF1022-producing microorganism.**

13. (Amended) A method for producing a cyclic depsipeptide synthetase, which  
comprises the steps of culturing the host of claim 8, and collecting the cyclic depsipeptide  
synthetase from the culture medium.

REMARKS

The specification has been amended to reflect the national stage status. In addition, the multiple dependencies of the claims have been modified to reduce the PTO filing fee and eliminate improper multiple dependencies.

The present application contains a Sequence Listing. The Sequence Listing has been submitted herewith in paper and computer readable form. The paper and computer readable forms of the Sequence Listing are identical and do not contain new matter.

Attached hereto is a marked-up version of the changes made to the specification and claims by the current amendment. The attached pages are captioned "Version with markings to show changes made".

Favorable action on the merits is solicited.

Respectfully submitted,

Naoki MIDOH et al.

By



Warren M. Cheek, Jr.  
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March 6, 2002

CYCLIC DEPSIPEPTIDE SYNTHETASE AND GENE THEREOF, AND MASS  
PRODUCTION SYSTEM FOR CYCLIC DEPSIPEPTIDE

This application is a 371 of PCT/JP00/06103 filed September 7, 2000.

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention relates to a cyclic depsipeptide synthetase and a gene thereof, and a mass production system for the cyclic depsipeptide. More specifically, the present invention relates to an enzyme for synthesizing substance PF1022 10 having anthelmintic activity and a gene thereof, and a mass production system for the substance PF1022.

Description of the Related Art

The substance PF1022 [cyclo(D-lactyl-L-N-methylleucyl-D-3-phenyllactyl-L-N-methylleucyl-D-lactyl-L-N-methylleucyl-D-3-phenyllactyl-L-N-methylleucyl)] is a cyclic depsipeptide which is produced by the filamentous fungus strain PF1022 (Mycelia sterilia, FERM BP-2671), which belongs to Agonomycetales, and has an extremely high anthelmintic activity against animal parasitic nematodes (Japanese Patent Application 20 Laid-open No. 35796/1991; Sasaki, T. et al., J. Antibiotics, 45, 692, 1992). Accordingly, this substance is useful as a anthelmintic and also as a raw material for synthesizing a highly active derivative of this substance.

Generally, the amount of secondary metabolites produced 25 by microorganisms isolated from nature is very small. Accordingly, in order to use the secondary metabolites industrially, it is necessary to improve the amount of the production. For this purpose, the culture method and the medium composition are investigated, fermentation conditions are 30 improved by addition of precursors and the like, and strains are improved by mutation with UV irradiation or mutation inducers. Recently, in addition to these means, genetic recombination technology has become available to improve the productivity.

For example, enhancement of expression of an enzyme gene 35 for biosynthesis, enhancement of expression of a regulatory gene for biosynthesis, and interruption of unnecessary biosynthesis pathways have been carried out (Khetan, A. and Hu, W.-S., Manual

(Amended)

7. A recombinant vector comprising the polynucleotide of  
~~any one of claims 2 to 6.~~ Claim 2 or Claim 4.

8. A host comprising the expression vector of claim 7.

9. The host according to claim 8, which expresses a cyclic  
depsipeptide synthetase.

(Amended)

10. The host according to claim 8 or 9, which is a substance  
PF1022-producing microorganism.

(Amended)

11. A method for producing a cyclic depsipeptide, which  
comprises the steps of culturing the host of claim 8, 9 or 10  
and collecting the cyclic depsipeptide from the culture medium.

12. The method according to claim 11, wherein the cyclic  
depsipeptide is the substance PF1022 and a derivative thereof.

(Amended)

13. A method for producing a cyclic depsipeptide  
synthetase, which comprises the steps of culturing the host of  
claim 8, 9 or 10 and collecting the cyclic depsipeptide synthetase  
from the culture medium.

14. The method according to claim 13, wherein the cyclic  
depsipeptide is the substance PF1022 and a derivative thereof.

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CYCLIC DEPSIPEPTIDE SYNTHETASE AND GENE THEREOF, AND MASS  
PRODUCTION SYSTEM FOR CYCLIC DEPSIPEPTIDE

BACKGROUND OF THE INVENTION

5 Field of the Invention

The present invention relates to a cyclic depsipeptide synthetase and a gene thereof, and a mass production system for the cyclic depsipeptide. More specifically, the present invention relates to an enzyme for synthesizing substance PF1022 10 having anthelmintic activity and a gene thereof, and a mass production system for the substance PF1022.

Description of the Related Art

The substance PF1022 [cyclo(D-lactyl-L-N-methylleucyl-D-3-phenyllactyl-L-N-methylleucyl-D-lactyl-L-N-methylleucyl-D-3-phenyllactyl-L-N-methylleucyl)] is a cyclic depsipeptide which is produced by the filamentous fungus strain PF1022 (Mycelia sterilia, FERM BP-2671), which belongs to Agonomycetales, and has an extremely high anthelmintic activity against animal parasitic nematodes (Japanese Patent Application Laid-open No. 35796/1991; Sasaki, T. et al., J. Antibiotics, 45, 20 692, 1992). Accordingly, this substance is useful as a anthelmintic and also as a raw material for synthesizing a highly active derivative of this substance.

Generally, the amount of secondary metabolites produced 25 by microorganisms isolated from nature is very small. Accordingly, in order to use the secondary metabolites industrially, it is necessary to improve the amount of the production. For this purpose, the culture method and the medium composition are investigated, fermentation conditions are 30 improved by addition of precursors and the like, and strains are improved by mutation with UV irradiation or mutation inducers. Recently, in addition to these means, genetic recombination technology has become available to improve the productivity.

For example, enhancement of expression of an enzyme gene 35 for biosynthesis, enhancement of expression of a regulatory gene for biosynthesis, and interruption of unnecessary biosynthesis pathways have been carried out (Khetan, A. and Hu, W.-S., Manual

of Industrial Microbiology and Biotechnology, 2nd edition, p. 717, 1999). Furthermore, a known particular example is a method for increasing productivity in which a hemoglobin gene of a microorganism is expressed in order to enhance oxygen utilization ability (Minas, W. et al., Biotechnol. Prog., 14, 561, 1998).

In improving productivity using gene recombination technology, the most common technique is augmentation of expression of an enzyme gene for biosynthesis. To apply this technique, it is essential that a method of transforming a microorganism has been established, that a promoter and a terminator utilizable for expression augmentation are available, that the biosynthesis pathway has been elucidated, and that the related genes have been isolated. As to the substance PF1022-producing microorganism, a foreign gene has been successfully introduced by transformation (WO97/00944); however, the gene for biosynthesis has not been isolated.

The substance PF1022 comprises a structure in which L-N-methylleucine, D-lactic acid and D-phenyllactic acid are bonded via ester bonds and amide bonds. In a producing microorganism, it is synthesized by a certain kind of a peptide-synthesizing enzyme from four molecules of L-leucine, two molecules of D-lactic acid, and two molecules of D-phenyllactic acid. Peptide-synthesizing enzymes are those which carry out biosynthesis of secondary metabolites of microorganisms, such as peptides, depsipeptides, lipopeptides, and peptide lactone, using amino acids and hydroxy acids as a substrate. Sequences of some peptide-synthesizing enzymes have been already elucidated (Marahiel, M.A. et al., Chem. Rev., 97, 2651, 1997). The reaction by this type of enzyme is entirely different from that in a system of synthesizing a protein by a ribosome using mRNA as a template. It is thought that a peptide-synthesizing enzyme has one domain for each substrate and each substrate is activated by ATP in this domain and bonded via phosphopantethenic acid in the domain, and these then form amide bonds or ester bonds by a catalytic action in the regions between each domain.

## SUMMARY OF THE INVENTION

An objective of the present invention is to provide an enzyme that synthesizes a cyclic depsipeptide, in particular the substance PF1022 (referred to as "cyclic depsipeptide synthetase" hereinafter).

Another objective of the present invention is to provide a gene that encodes the cyclic depsipeptide synthetase (referred to as "cyclic depsipeptide synthetase gene" hereinafter).

Still another objective of the present invention is to provide a recombinant vector and a transformant for expressing the cyclic depsipeptide synthetase, a mass-production system of the cyclic depsipeptide, and a method of producing the cyclic depsipeptide using the system.

Another objective of the present invention is to provide a method for producing the cyclic depsipeptide synthetase.

A cyclic depsipeptide synthetase according to the present invention is a protein comprising an amino acid sequence selected from the group consisting of the following sequences:

(a) an amino acid sequence of SEQ ID NO: 2, and  
20 (b) a modified amino acid sequence of the amino acid sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has cyclic depsipeptide synthetase activity.

A cyclic depsipeptide synthetase gene according to the present invention comprises a nucleotide sequence encoding the cyclic depsipeptide synthetase.

Further, a cyclic depsipeptide synthetase gene according to the present invention comprises a nucleotide sequence selected from the group consisting of the following sequences:

30 (c) a DNA sequence of SEQ ID NO: 1,  
(d) a nucleotide sequence that has at least 70% homology to the DNA sequence of SEQ ID NO: 1 and encodes a protein having cyclic depsipeptide synthetase activity,  
35 (e) a modified DNA sequence of the DNA sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and encodes a protein having cyclic depsipeptide synthetase

activity, and

(f) a nucleotide sequence that hybridizes with the DNA sequence of SEQ ID NO: 1 under stringent conditions and encodes a protein having cyclic depsipeptide synthetase activity.

5 A recombinant vector according to the present invention comprises a cyclic depsipeptide synthetase gene according to the present invention.

A transformant and a mass-production system of the cyclic depsipeptide according to the present invention are a host 10 comprising the recombinant vector according to the present invention.

A method for producing the cyclic depsipeptide according to the present invention comprises the steps of culturing the transformant according to the present invention and collecting 15 the cyclic depsipeptide from the culture medium.

A method for producing a cyclic depsipeptide synthetase comprises the steps of culturing the transformant according to the present invention and collecting the cyclic depsipeptide synthetase from the culture medium.

20 According to the present invention, a cyclic depsipeptide synthetase can be excessively expressed in a substance PF1022-producing microorganism, and the substance PF1022 can be massively produced.

25 BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows a construction procedure of plasmid pABP/PFsyn.

Figure 2 shows a restriction map of a 6 kb HindIII fragment comprising the Abp1 gene.

30 Figure 3 shows the construction and restriction map for pABPd.

Figure 4 shows the results of electrophoresis of the proteins extracted from the parent strain and a gene-introduced strain into which pABP/PFsyn is introduced.

35 Figure 5 shows the results of electrophoresis of the proteins extracted from the parent strain and a gene-introduced strain into which pABP/PFsynN is introduced.

## DETAILED DESCRIPTION OF THE INVENTION

Deposition of microorganisms

The strain PF1022 described in Example 1-1 was deposited  
5 with the National Institute of Bioscience and Human-Technology,  
Agency of Industrial Science and Technology, the Ministry of  
International Trade and Industry (1-3 Higashi 1-Chome, Tsukuba  
City, Ibaraki Prefecture, Japan), dated January 24, 1989. The  
accession number is FERM BP-2671.

10 *Escherichia coli* (DH5 $\alpha$ ) transformed with plasmid pPFsyn  
described in Example 2-1-(1) was deposited with the National  
Institute of Bioscience and Human-Technology, Agency of  
Industrial Science and Technology, the Ministry of International  
Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki  
15 Prefecture, Japan), dated September 1, 1999. The accession  
number is FERM BP-7253.

20 *Escherichia coli* (DH5 $\alpha$ ) transformed with plasmid pPFsynN  
described in Example 2-1-(1) was deposited with the National  
Institute of Bioscience and Human-Technology, Agency of  
Industrial Science and Technology, the Ministry of International  
Trade and Industry (1-3 Higashi 1-Chome, Tsukuba City, Ibaraki  
Prefecture, Japan), dated September 1, 1999. The accession  
number is FERM BP-7254.

Gene and protein

25 The present invention provides a cyclic depsipeptide  
synthetase, preferably a substance PF1022-synthesizing enzyme,  
and a gene thereof.

30 The enzyme according to the present invention acts on four  
molecules of L-leucine, two molecules of D-lactic acid, and two  
molecules of D-phenyllactic acid to synthesize substance PF1022.  
A derivative of substance PF1022 can be produced by in advance  
modifying D-lactic acid, L-leucine, and D-phenyllactic acid.

35 Examples of derivatives of substance PF1022 are  
derivatives in which two phenyl groups at the para positions in  
substance PF1022 are substituted by amino groups. In this case,  
for example, D-p-amino phenyllactic acid can be used instead of  
D-phenyllactic acid as a synthesizing substrate for the substance

PF1022 derivative.

In sequence (b), the number of modifications can be, for example, one to several, more specifically, 1 to 6.

5 In sequence (e), the number of modifications can be, for example, one to dozens.

In sequence (b) and sequence (e), if multiple mutations are introduced, said mutations can be the same or different.

Sequence (d) can be preferably at least 80%, more preferably at least 90%, or most preferably at least 95% homology 10 to the DNA sequence of SEQ ID NO: 1.

15 In sequence (f), the term "stringent conditions" means that a membrane after hybridization is washed at a high temperature in a solution of low salt concentration, for example, at a 0.2 x SSC concentration (1 x SSC: 15 mM trisodium citrate, 150 mM sodium chloride) in a 0.1% SDS solution at 60°C for 15 minutes.

Whether sequence (b) "has the cyclic depsipeptide synthetase activity" or not can be evaluated, for example, by providing a substrate for the cyclic depsipeptide, reacting the protein to be tested, and then confirming the synthesis of the 20 cyclic depsipeptide, for example, by chromatography.

Whether sequences (d), (e) and (f) "encode a protein having cyclic depsipeptide synthetase activity" or not can be evaluated, for example, by expressing the nucleotide sequence to be tested in a host, reacting the resulting protein with a substrate for 25 the cyclic depsipeptide, and then confirming the synthesis of the cyclic depsipeptide, for example, by chromatography, as described in Example 2.

Given the amino acid sequence of a synthesizing enzyme of the present invention, nucleotide sequences encoding the amino 30 acid sequence can be easily determined, and various nucleotide sequences encoding the amino acid sequence depicted in SEQ ID NO: 2 can be selected. Thus, nucleotide sequences encoding the synthesizing enzyme according to the present invention include any DNA sequence encoding the same amino acid sequence and having 35 degenerative codons, in addition to a part or all of the DNA sequence of SEQ ID NO: 1, and further includes RNA sequences corresponding to those sequences.

A gene according to the present invention can be obtained, for example, based on the following.

A genomic DNA is isolated from a substance PF1022-producing microorganism and cleaved with appropriate restriction enzymes, 5 and a library comprising the genomic DNA of the substance PF1022-producing microorganism is constructed using a phage vector. Appropriate primers are synthesized based on a conservative region of the amino acid sequence of the peptide-synthesizing enzyme or a partial amino acid sequence of 10 the cyclic peptide-synthesizing enzyme purified from the substance PF1022-producing microorganism. The PCR method is carried out using the primers and the genomic DNA derived from the substance PF1022-producing microorganism as a template, and thus the DNA fragment of the cyclic peptide-synthesizing enzyme 15 gene is amplified. The genomic library is screened using this DNA fragment as a probe. Thus, the whole region of the cyclic peptide-synthesizing enzyme gene can be isolated. After determining the nucleotide sequence of this DNA fragment, appropriate restriction enzyme cleavage sites are introduced 20 upstream of the translation start codon and downstream of the translation stop codon by PCR or the like to obtain a gene fragment which contains the cyclic depsipeptide synthetase gene, exclusively.

Recombinant vector

25 The present invention provides a recombinant vector comprising a nucleotide sequence encoding a cyclic depsipeptide synthetase.

The procedure and method for constructing a recombinant vector according to the present invention can be any of those 30 commonly used in the field of genetic engineering.

Examples of the vector to be used in the present invention include vectors that can be incorporated into a host chromosome DNA and vectors having a self-replicable autonomous replication sequence which can be present as a plasmid in a host cell, for 35 example, pUC vectors (e.g., pUC18 and pUC118), pBluescript vectors (e.g., pBluescriptII KS+), and pBR322 plasmid. One or more copies of the gene can be present in a host cell.

A recombinant vector according to the present invention can be constructed, for example, by operably ligating a promoter and a terminator upstream and downstream of the nucleotide sequence encoding a cyclic depsipeptide synthetase, respectively, 5 and if appropriate, a gene marker and/or other regulatory sequences.

The ligation of the promoter and terminator to the gene according to the present invention and the insertion of the expression unit into the vector can be carried out by known 10 methods.

A promoter and a terminator to be used in the present invention are not particularly limited. Examples of the promoter and the terminator include regulatory sequences of genes of glycolysis enzymes, such as 3-phosphoglycerate kinase, 15 glyceraldehyde-3-phosphate dehydrogenase and enolase; regulatory sequences of amino acid-synthetase genes, such as ornithine carbamoyltransferase and tryptophan synthase; regulatory sequences of hydrolase genes, such as amylase, protease, lipase, cellulase, and acetamidase; regulatory 20 sequences of genes of oxidation-reduction enzymes, such as nitrate reductase, orotidine-5'-phosphate dehydrogenase, and alcohol dehydrogenase; and a regulatory sequence of a gene derived from a substance PF1022-producing microorganism, such as Abpl, which is highly expressed in the substance PF1022-producing 25 microorganism.

A protein of the present invention can be expressed as a fusion protein by ligating a gene according to the present invention to a foreign gene encoding a translation region of another protein.

30 A gene marker can be introduced, for example, by introducing an appropriate restriction enzyme cleaving site into a regulatory sequence by the PCR method, inserting this regulatory sequence into a plasmid vector, and ligating a selective marker gene such as a drug resistance gene and/or a gene complementing 35 a nutritional requirement to the vector.

A gene marker can be appropriately selected depending on the technique for selecting a transformant. For example, a gene

encoding drug resistance or a gene complementing a nutritional requirement can be used. Examples of the drug resistance gene include genes conferring resistance to destomycin, benomyl, oligomycin, hygromycin, G418, bleomycin, bialaphos, blastcidin S, phleomycin, phosphinothricin, ampicillin, and kanamycin.

5 Examples of the gene complementing a nutritional requirement include amdS, pyrG, argB, trpC, niaD, TRP1, LEU2, URA3, and the like.

Production of transformant and cyclic depsipeptide

10 The present invention provides a host transformed with the above-mentioned vector.

A host to be used in the present invention is, not particularly restricted, any microorganism which can be used as a host for genetic recombination. Examples of the host to be used

15 include microorganisms, namely certain bacteria or fungi, preferably Escherichia coli, bacteria of genus Bacillus, actinomycetes, yeasts, and filamentous fungi, more preferably filamentous fungi which produce substance PF1022, most preferably the strain PF1022 (Mycelia sterilia, FERM BP-2671).

20 A recombinant vector for the gene expression can be introduced into a host by an ordinary method. Examples of the method for the introduction include the electroporation method, the polyethylene glycol method, the Agrobacterium method, the lithium method, and the calcium chloride method. A method most effective for a specific host cell can be selected. The polyethylene glycol method is preferable when a substance PF1022-producing microorganism is used as a host.

25 A transformant can be cultured by appropriately selecting a medium, culture conditions and the like according to an ordinary method. Conventional components can be used for a medium. As a carbon source, glucose, sucrose, cellulose, starch syrup, dextrin, starch, glycerol, molasses, animal and vegetable oils, and the like can be used. As a nitrogen source, soybean powder, wheat germ, pharma media, cornsteep liquor, cotton seed lees,

30 bouillon, peptone, polypeptone, malt extract, yeast extract, ammonium sulfate, sodium nitrate, urea, and the like can be used. If necessary, inorganic salts which can produce sodium, potassium,

calcium, magnesium, cobalt, chlorine, phosphoric acid, sulfuric acid, and other ions, such as potassium chloride, calcium carbonate, dipotassium hydrogenphosphate, magnesium sulfate, monopotassium phosphate, zinc sulfate, manganese sulfate, and copper sulfate, can be effectively added. If necessary, various vitamins such as thiamine (e.g., thiamine hydrochloride), amino acids such as glutamic acid (e.g., sodium glutamate) and asparagine (e.g., DL-asparagine), trace nutrients such as nucleotides, and selective drugs such as antibiotics can be added.

Further, organic and inorganic substances to promote microbial growth and enhance cyclic depsipeptide production can be appropriately added.

The cultivation can be carried out by a shaking culture method under an aerobic condition, an agitation culture method with aeration, or an aerobic submerged culture method. In particular, an aerobic submerged culture method is most preferable. The pH of the medium is, for example, about 6 to 8. An appropriate culture temperature is 15°C to 40°C. Most cells grow at about 26°C to 37°C. Production of the cyclic depsipeptide synthetase and cyclic depsipeptide depends on a media, culture conditions, or a host used. However, the maximum accumulation can be attained generally in 2 to 25 days in any culture method.

The cultivation is terminated when the amount of the cyclic depsipeptide synthetase or cyclic depsipeptide in the medium reaches its peak, and the cyclic depsipeptide synthetase or cyclic depsipeptide is isolated from the culture and purified.

The cyclic depsipeptide synthetase or cyclic depsipeptide can be extracted and purified from the culture by any conventional separation method based on its properties, such as a solvent extraction method, an ion-exchange resin method, adsorption or distribution column chromatography, gel filtration, dialysis, precipitation, and crystallization, either singly or appropriately in combination.

The cyclic depsipeptide synthetase can be efficiently purified by hydrophobic chromatography using butyl agarose or the like.

The cyclic depsipeptide can be extracted from the culture,

for example, with acetone, methanol, butanol, ethyl acetate, or butyl acetate. The cyclic depsipeptide can be further purified by chromatography using an adsorbent such as silica gel and aluminum, Sephadex LH-20 (Pharmacia), or Toyopearl HW-40 (Toso Co.). The pure cyclic depsipeptide can be obtained by using any of above-mentioned methods, either singly or appropriately in combination.

The present invention provides a mass production system of a cyclic depsipeptide. A host applicable to a cyclic depsipeptide production system, particularly a substance PF1022 production system, is preferably a substance PF1022-producing filamentous fungus, most preferably the strain PF1022 (Mycelia sterilia, FERM BP-2671). A recombinant vector used for transformation is preferably an expression vector in which a regulatory sequence (e.g., promoter and terminator), which functions in the substance PF1022-producing microorganism, is operably linked to a cyclic depsipeptide synthetase gene, most preferably an expression vector in which a regulatory sequence, which functions in the strain PF1022 (Mycelia sterilia, FERM BP-2671), is operably linked to a cyclic depsipeptide synthetase gene. A cyclic depsipeptide, particularly the substance PF1022, can be preferably produced by culturing a substance PF1022-producing microorganism transformed with an expression vector in which a regulatory sequence, which functions in the substance PF1022-producing microorganism, is operably linked to a cyclic depsipeptide synthetase gene, and isolating the cyclic depsipeptide from the culture.

In a host that does not synthesize any substrate for the substance PF1022, i.e., L-leucine, D-lactic acid or D-phenyllactic acid, the deficient substrate or a derivative thereof can be added to a culture medium to produce the substance PF1022 or a derivative thereof.

#### EXAMPLE

The present invention will now be illustrated in detail with reference to the following examples; however, these examples are not construed to limit the scope of the invention.

Example 1: Cloning of cyclic depsipeptide synthetase gene from substance PF1022-producing microorganism

1. Isolation of genomic DNA and construction of genomic library

The strain PF1022 (*Mycelia sterilia*, FERM BP-2671) was subjected to UV radiation or NTG treatment to induce mutation, and a genomic DNA was extracted from the resulting substance PF1022-producing strain 432-26, in which PF1022 productivity was improved. The substance PF1022-producing strain 432-26 was cultured in 50 ml of a seed medium (1% yeast extract, 1% malt extract, 2% polypeptone, 2.5% glucose, 0.1% dipotassium hydrogenphosphate, 0.05% magnesium sulfate, pH 7.0) at 26°C for 2 days, and the cells were recovered by centrifugation. The cells thus obtained were frozen with liquid nitrogen and then smashed with a mortar and pestle. A genomic DNA was isolated from these smashed cells using ISOPLANT (Nippon Gene Co., Ltd.) according to the attached protocol. The isolated genomic DNA was partially digested with Sau3AI, after which a DNA fragment of 15 kb to 20 kb was recovered by agarose gel electrophoresis and treated with alkaline phosphatase to dephosphorylate the terminal of the DNA fragment. This DNA fragment was inserted into a phage vector, Lambda DASH II (Stratagene Co.). The recombinant phage vector thus obtained was subjected to in vitro packaging using a GigapackIII Gold Packaging Extract (Stratagene Co.) according to the attached protocol. Thereafter, *Escherichia coli* XL1-Blue MRA (P2) strain was infected with this recombinant phage and cultured on a plate to form a plaque.

2. Isolation of partial DNA fragment of cyclic depsipeptide synthetase gene

Multiple alignment of a known peptide-synthesizing enzyme was carried out, and well-conserved regions, WTSMYDG (SEQ ID NO: 3) and VVQYFPT (SEQ ID NO: 4) were found. Primers 5'-TGGACIWSNATGTAYGAYGG-3' (SEQ ID NO: 5) and 5'-GTIGGRAARTAYTGIACNAC-3' (SEQ ID NO: 6) were synthesized based on these sequences. Using these primers, PCR was carried out using the genomic DNA isolated from the substance PF1022-producing microorganism as a template. The PCR was conducted in 50 µl of reaction solution using 50 ng of the genomic DNA as a

template, 1.25 units of ExTaq DNA polymerase (Takara Shuzo Co., Ltd.), attached buffer and dNTP Mixture, and 10  $\mu$ M primer, under the following conditions: 3 minutes at 94°C; 30 cycles of [one minute at 94°C, one minute at 65°C (lowered 0.5°C per cycle), one minute at 72°C]; and 3 minutes at 72°C. A DNA fragment of about 350 bp was amplified by this reaction and this DNA fragment was inserted into plasmid vector pCR2.1 using an Original TA Cloning Kit (Invitrogen) according to the attached protocol.

The nucleotide sequence of the DNA fragment thus cloned 10 was determined using a DNA Sequencing Kit dRhodamine Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the attached protocol. Results revealed that the nucleotide sequence of the isolated DNA fragment was homologous to that of 15 the peptide synthesizing enzyme gene, and was a part of the cyclic depsipeptide synthetase gene of interest.

### 3. Cloning of the whole region of cyclic depsipeptide synthetase gene

A probe used in the screening of the genomic library was 20 prepared by incorporating fluorescein-labeled dUTP into a DNA fragment by PCR. The PCR was carried out in 50  $\mu$ l of reaction solution using plasmid vector pCR2.1, into which 100 ng of the cyclic depsipeptide synthetase gene DNA fragment was inserted, as a template, 1.25 units of ExTaq DNA polymerase (Takara Shuzo, 25 Co., Ltd.) and attached buffer, 0.2 mM dATP, 0.2 mM dCTP, 0.2 mM dGTP, 0.02 mM dTTP, 0.18 mM fluorescein-labeled dUTP (FluoroGreen, Amersham Pharmacia Biotech), and 10  $\mu$ M primers (SEQ ID NO: 5 and SEQ ID NO: 6) under the following conditions: 2 minutes 30 at 94°C; 25 cycles of [30 seconds at 94°C, one minute at 55°C, one minute at 72°C]; and 3 minutes at 72°C. A fluorescein-labeled probe of about 350 bp was constructed by this reaction.

A Hibond-N+ Membrane (Amersham Pharmacia Biotech) was placed on a plate with the plaque, which was prepared in Example 1-1, to adhere the plaque. The membrane was treated with alkaline, 35 the recombinant phage DNA on the membrane was denatured into a single strand and adsorbed to the membrane. The membrane with the adsorbed phage DNA was taken into a buffer solution prepared

using Hybridization Buffer Tablets (Amersham Pharmacia Biotech), and then incubation was carried out at 60°C for 1 hour. The above-mentioned fluorescein-labeled probe was denatured by heat and added to this, and hybridization was carried out at 60°C overnight. The membrane was then washed in a 1 x SSC (SSC: 15 mM trisodium citrate, 150 mM sodium chloride) - 0.1% SDS solution at 60°C for 15 minutes, and further in a 0.2 x SSC - 0.1% SDS solution at 60°C for 15 minutes. The fluorescein-bonded plaque was visualized using a DIG Wash and Block Buffer Set (Boehringer-Manheim), anti-fluorescein antibody labeled with alkaline phosphatase (Anti-fluorescein-AP, Fab fragment; Boehringer-Manheim), and nitroblue tetrazolium chloride (Boehringer-Manheim) and X-phosphate (Boehringer-Manheim) as coloring substrates, according to the attached protocol. In this way, a positive clone containing a 5' upstream region and a 3' downstream region homologous to the probe was selected.

#### 4. Determination of nucleotide sequence

The DNA fragment in the positive clone thus isolated was amplified by PCR using phage vector sequences 5'-GCGGAATTAAACCTCACTAAAGGGAACGAA-3' (SEQ ID NO: 7) and 5'-GCGTAATACGACTCACTATAGGGCGAAGAA-3' (SEQ ID NO: 8) as primers. The PCR was carried out in 50 µl of reaction solution using 100 ng of the positive clone DNA as a template, 2.5 units of LA Taq DNA polymerase (Takara Shuzo Co., Ltd.), attached buffer and dNTP mixture, 2.5 mM magnesium chloride, and 0.2 µM primers under the following conditions: one minute at 94°C; 25 cycles of [10 seconds at 98°C, 15 minutes at 68 °C]; and 15 minutes at 72°C. The resulting PCR product was purified, treated with a nebulizer and randomly decomposed to 0.5 kb to 2.0 kb. The terminals of these fragments were blunted with T4 DNA polymerase and phosphorylated with T4 polynucleotide kinase, after which the resulting fragments were inserted into the EcoRV site of pT7Blue (Novagen, inc.) and introduced into Escherichia coli JM109 strain. The resulting 168 colonies were subjected to direct PCR using M13 Primer M4 (Takara Shuzo Co., Ltd.) and M13 Primer RV (Takara Shuzo Co., Ltd.). After purification, sequencing was carried out using M13 Primer M4 (Takara Shuzo Co., Ltd.) as a primer. The PCR was carried out

in 50 µl of reaction solution using 1.25 units of ExTaq DNA polymerase (Takara Shuzo Co., Ltd.), attached buffer and dNTP Mixture, and 0.5 µM primers, under the following conditions: 4 minutes at 94°C; 30 cycles of [30 seconds at 94°C, 30 seconds at 5 55°C, 2 minutes at 72°C); and 3 minutes at 72°C. Sequencing was carried out using a DNA Sequencing Kit dRhodamine Terminator Cycle Sequencing Ready Reaction (Applied Biosystems) and an ABI PRISM 310 Genetic Analyzer (Applied Biosystems) according to the attached protocol.

10 From the result, regions where the analysis was not sufficient were amplified by PCR with primers newly designed based on already-analyzed nucleotide sequences, and after purification, sequencing was carried out using the primers used for the PCR. The nucleotide sequence of the 15606-bp DNA fragment in the 15 positive clone was determined.

15 The analysis of this sequence revealed that a 9633-bp open reading frame (ORF) was present and that the protein extrapolated from this sequence had 3210 amino acid residues and was 354 kDa and homologous to peptide-synthesizing enzymes. The enniatin-synthesizing enzyme (S39842) showed the highest homology, namely 56%. The nucleotide sequence and the amino acid sequence of the ORF of the cyclic depsipeptide synthetase gene of the present invention thus isolated are shown in SEQ ID NO: 1 and SEQ ID NO: 2, respectively.

20 Example 2: Improvement of PF1022 productivity by excessive expression of cyclic depsipeptide synthetase gene

25 1. Construction of recombinant vector for gene expression (Figure 1)

(1) Cloning of cyclic depsipeptide synthetase gene region

30 The inserted DNA fragment was cleaved with NotI from the positive clone obtained in Example 1-3, and inserted into the NotI site of pBluescriptII KS+ (Stratagene Co.) to construct plasmid pPF7. The pPF7 was cleaved with BanIII and SmaI, after which agarose gel electrophoresis was carried out and a DNA 35 fragment of about 8250 bp was recovered from the agarose gel. This fragment was inserted into pBluescriptII KS+ to construct plasmid pPF7-1.

PCR was carried out using pPF7 as a template; 5'-AGCATGGATCCTAACAAATGGCGTTGAGCAGCAAGCCCTA-3' (SEQ ID NO: 9, designed to start translation from Met on position 10 from the N-terminal of the ORF) or 5'-AGCATGGATCCTAACAAATGTCAAACATGGCACCACTCCCTA-3' (SEQ ID NO: 11, designed to start translation from Met on position 1 from the N-terminal of the ORF), and 5'-TTTGCTTCGTACTCGGGTCCT-3' (SEQ ID NO: 10) as primers for the amplification of the fragment of about 440 bp (SEQ ID NO: 9 and SEQ ID NO: 10 were used) or the fragment of about 470 bp (SEQ ID NO: 11 and SEQ ID NO: 10 were used) from near the N-terminal to the BanIII site; and further, 5'-GCATCGCGATACTAGAGAAG-3' (SEQ ID NO: 12) and 5'-AGCATCGAATTGGATCCCTAACCAACGCCAAGCCCCGAAT-3' (SEQ ID NO: 13) as primers for the amplification of the fragment of about 920 bp from the SmaI site to the C-terminal. Here, the primers were designed to introduce BamHI sites to the 5' and 3' sides of the cyclic depsipeptide synthetase gene of the present invention. The PCR was carried out in 50 µl of reaction solution using 150 ng of plasmid DNA as a template, 2.5 units of KOD DNA polymerase (Toyobo Co., Ltd.) and attached buffer and dNTP Mixture, 1 mM magnesium chloride, and 0.5 µM primers, under the following conditions: 30 seconds at 98°C; 10 cycles of [15 seconds at 98°C, 2 seconds at 65°C, 30 seconds at 74°C]; and one minute at 74°C. The PCR reaction solution obtained using each primer was precipitated with ethanol to recover PCR products. The N-terminal region was cleaved with BamHI and BanIII, and the C-terminal region was cleaved with SmaI and BamHI, after which agarose gel electrophoresis was carried out to recover DNA fragments from the agarose gel.

The above-mentioned C-terminal region PCR fragment was inserted into the SmaI, BamHI sites of pPF7-1 to construct plasmid pPF7-2. This plasmid was cleaved with BanIII and BamHI, after which agarose gel electrophoresis was carried out to recover a DNA fragment of about 9170 bp from the agarose gel. This DNA fragment and the N-terminal region prepared using SEQ ID NO: 9 and SEQ ID NO: 10 were simultaneously inserted into the BamHI site of pBluescriptII KS+ to reconstruct the cyclic depsipeptide

synthetase gene of the present invention, and thus plasmid pPFsyn (in which translation starts from Met on position 10 from the N-terminal of ORF) was constructed.

On the other hand, the DNA fragment of about 9170 bp cleaved 5 from pPF7-2 and the N-terminal region constructed using SEQ ID NO: 9 and SEQ ID NO: 11 were simultaneously inserted into the BamHI site of pHSG299 (Takara Shuzo Co., Ltd.) to reconstruct the cyclic depsipeptide synthetase gene of the present invention, and thus plasmid pPFsynN (in which translation starts from Met 10 on position 1 from the N-terminal of ORF) was constructed. In this way, the cyclic depsipeptide synthetase gene having BamHI sites on both terminals was constructed.

After cleaving pPFsyn or pPFsynN with BamHI, the cyclic depsipeptide synthetase gene region was each recovered from the 15 gel.

(2) Construction of expression vector using expression regulatory region of Abp1 gene

Isolation of genomic DNA of substance PF1022-producing microorganism

The genomic DNA of the substance PF1022-producing 20 microorganism (FERM BP-2671) was isolated according to the method of Horiuchi et al. (H. Horiuchi et al., J. Bacteriol., 170, 272-278, 1988). More specifically, cells of the substance PF1022-producing strain (FERM BP-2671) were cultured for 2 days in a 25 seed medium (2.0% soluble starch, 1.0% glucose, 0.5% polypeptone, 0.6% wheat germ, 0.3% yeast extract, 0.2% soybean cake, and 0.2% calcium carbonate; pH 7.0 before sterilization; see Example 1 in WO97/00944), and the cells were recovered by centrifugation (3500 rpm, 10 minutes). The cells thus obtained were lyophilized, 30 suspended in a TE solution, treated in a 3% SDS solution at 60°C for 30 minutes, and then subjected to TE-saturated phenol extraction to remove the cell residue. The extract was precipitated with ethanol and treated with Ribonuclease A (Sigma) and Proteinase K (Wako Pure Chemical Industries, Ltd.), and the 35 nucleic acid was then precipitated with 12% polyethylene glycol 6000. The precipitate was subjected to TE-saturated phenol extraction and ethanol precipitation, and the resulting

precipitate was dissolved in a TE solution to obtain the genomic DNA.

Construction of genomic library of substance PF1022-producing microorganism

5       The genomic DNA derived from the substance PF1022-producing microorganism prepared as described above was partially digested with Sau3AI. The product was ligated to the BamHI arm of a phage vector, a  $\lambda$ EMBL3 Cloning kit (Stratagene Co.) using T4 ligase (Ligation Kit Ver. 2; Takara Shuzo Co., Ltd.).

10      After ethanol precipitation, the precipitate was dissolved in a TE solution. The entire ligated mixture was used to infect Escherichia coli LE392 strain using a Gigapack III Plus Packaging kit (Stratagene Co.) to form phage plaques. The  $1.3 \times 10^4$  ( $2.6 \times 10^4$  PFU/ml) phage library obtained by this method was used for

15      cloning of the Abp1 gene.

Cloning of the Abp1 gene from the genomic DNA derived from substance PF1022-producing microorganism

A probe to be used was prepared by amplifying the translation region of the Abp1 gene by the PCR method. The PCR was carried out using the genomic DNA prepared from the substance PF1022-producing microorganism as described above as a template and synthetic primers 8-73U and 8-73R, according to a LETS GO PCR kit (SAWADY Technology). The PCR reaction for amplification was conducted by repeating 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 90 seconds at 72°C. DNA sequences of the 8-73U and 8-73R are as follows:

8-73U: CACAAACCAGGAACCTTTTC (SEQ ID NO: 14)

8-73R: GACATGTGGAAACCACATTTG (SEQ ID NO: 15)

The PCR product thus obtained was labeled using an ECL Direct System (Amersham Pharmacia Biotech). The phage plaque prepared as described above was transferred to a Hibond N+ nylon transfer membrane (Amersham Pharmacia Biotech), and after alkaline denaturation, the membrane was washed with 5-fold concentration SSC (SSC: 15 mM trisodium citrate, 150 mM sodium chloride), and dried to immobilize the DNA. According to the kit protocol, prehybridization (42°C) was carried out for 1 hour, after which the previously labeled probe was added, and

hybridization was carried out at 42°C for 16 hours. The probe was washed according to the kit protocol described above. The nylon membrane with the washed probe was immersed for one minute in a detection solution, and was then photosensitized on a medical 5 x-ray film (Fuji Photo Film Co., Ltd.) to obtain one positive clone. Southern blot analysis of this clone showed that a HindIII fragment of at least 6 kb was identical with the restriction enzyme 10 fragment of the genomic DNA. Figure 2 shows the restriction map of this HindIII fragment. The HindIII fragment was subcloned 15 into pUC119 to obtain pRQHin/119 for the following experiments.

Construction of expression vector

The promoter region and the terminator region of the Abp1 gene were amplified by the PCR method using pRQHin/119 as a template. The PCR was carried out using a PCR Super Mix High 15 Fidelity (Lifetech Oriental Co., Ltd.), primers ABP-Neco and ABP-Nbam for promoter amplification and ABP-Cbam and ABP-Cxba for terminator amplification. The amplification reaction was conducted by repeating 25 cycles of 30 seconds at 94°C, 30 seconds at 50°C, and 90 seconds at 72°C. The DNA sequences of ABP-Neco, 20 ABP-Nbam, ABP-Cbam and ABP-Cxba are as follows:

ABP-Neco: GGGGAATTCGTGGTGGTGATATCATGGC (SEQ ID NO: 16)

ABP-Nbam: GGGGGATCCTTGATGGGTTTGGG (SEQ ID NO: 17)

ABP-Cbam: GGGGGATCCTAAACTCCCATCTATAGC (SEQ ID NO: 18)

ABP-Cxba: GGGTCTAGACGACTCATTGCAGTGAGTGG (SEQ ID NO: 19)

25 Each PCR product was purified with a Microspin S-400 column (Amersham Pharmacia Biotech) and precipitated with ethanol, after which the promoter was digested with EcoRI and BamHI, the terminator was digested with BamHI and XbaI, and the resulting fragments were ligated one by one to pBluescriptII KS+ previously 30 digested with the same enzymes. The product was digested with XbaI, and a destomycin resistance cassette derived from pMKD01 (WO98/03667) was inserted to construct pABPd (Figure 3). The pABPd has the promoter and the terminator of the Abp1 gene.

35 The cyclic depsipeptide synthetase gene region recovered from the gel as described above was inserted into the BamHI site of pABPd to construct expression vectors for the expression of the cyclic depsipeptide synthetase gene, i.e., pABP/PFsyn (in

which translation starts from Met on position 10 from the N-terminal of ORF) and pABP/PFsynN (in which translation starts from Met on position 1 from the N-terminal of ORF).

2. Introduction of cyclic depsipeptide synthetase gene into substance PF1022-producing microorganism, and expression of the gene

The expression vector was introduced into the strain PF1022 (*Mycelia sterilia*, FERM BP-2671) according to the method described in Example 1 of WO97/00944, and strains having a high hygromycin B resistance were selected. The introduction of the gene of interest in these strains was verified by PCR using the primer 5'-TGATATGCTGGAGCTTCCCT-3' (SEQ ID NO: 20) constructed from the sequence of the *Abp1* promoter, and the primer 5'-GCACAAACCTCTTCCAGGCT-3' (SEQ ID NO: 21) constructed from the sequence of the cyclic depsipeptide synthetase gene. Thus, gene-introduced strains having a high hygromycin B resistance, into which the cyclic depsipeptide synthetase gene of the present invention was introduced, were selected.

The gene-introduced strains and the parent strain (*Mycelia sterilia*, FERM BP-2671) were each cultured in 50 ml of the seed medium at 26°C for 2 days, after which a 1 ml portion of each resultant culture was inoculated into 50 ml of a production medium (6% starch syrup, 2.6% starch, 2% wheat germ, 1% pharma media, 0.2% magnesium sulfate heptahydrate, 0.2% calcium carbonate, and 0.3% sodium chloride; pH 7.5), and the cultivation was carried out at 26°C for 4 days. The resulting culture was centrifuged at 4500 rpm for 5 minutes to recover the cells. The cells thus obtained were washed with 0.3 M potassium chloride, frozen with liquid nitrogen, and then lyophilized.

The extraction procedure described below was carried out on ice or in a cold room at 4°C. The lyophilized cells (10 mg) and 1.0 ml of glass beads (0.5 mm in diameter) were placed in a 2 ml-microtube, and 1.0 ml of extraction buffer [50 mM Tris-HCl (pH 8.0), 0.3 M potassium chloride, 60% glycerol, 10 mM ethylenediamine disodium tetraacetate, 5 mM dithiothreitol, 10 μM leupeptin, 1 mM phenylmethanesulfonic acid, 60 μg/ml chymostatin] was added into the microtube. This microtube was

set on a Mini-Bead Beater-8 (Biospeck) and extraction was carried out at the maximum speed for 3 minutes. After centrifugation at 15000 rpm for 5 minutes, 100 µl of supernatant was admixed with 100 µl of 10% trichloroacetic acid solution. After allowing to stand for 15 minutes, the admixture was centrifuged at 15000 rpm for 10 minutes, and the resultant precipitate was dissolved in 15 µl of an alkaline solution (2% sodium carbonate, 0.4% sodium hydroxide), and 60 µl of sample buffer [125 mM Tris-HCl (pH 6.8), 20% glycerol, 4% sodium dodecyl sulfate, 10% 2-mercapto ethanol, 10 50 mg/L Bromophenol Blue] was added. The resultant admixture was heated in boiling water for 5 minutes, and then subjected to electrophoresis with 4% to 20% polyacrylamide gels [sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)] using an electrophoresis system (Tefco). After electrophoresis, 15 the polyacrylamide gels were stained with Quick-CBB (Wako Pure Chemical Industries, Ltd.) according to the attached protocol. The result of the electrophoresis of the proteins extracted from the parent strain and the gene-introduced strain into which pABP/PFsyn was introduced is shown in Figure 4. The result of 20 the electrophoresis of the proteins extracted from the parent strain and the gene-introduced strains into which pABP/PFsynN was introduced is shown in Figure 5.

Thus, the cyclic depsipeptide synthetase of the gene-introduced strains was much more highly expressed than that of 25 the parent strain.

### 3. Extraction and quantitative measurement of substance PF1022

The gene-introduced strains and the parent strain were each cultured in 50 ml of the seed medium at 26°C for 2 days, after 30 which a 1 ml portion of each culture was inoculated into 50 ml of the production medium, and the cultivation was carried out at 26°C for 6 days. A 10 ml portion of each resulting culture was centrifuged at 3000 rpm for 10 minutes to recover the cells. Methanol (10 ml) was added to the cells, and the admixture was vigorously shaken and then allowed to stand for 30 minutes. The 35 admixture was shaken again and centrifuged at 3000 rpm for 10 minutes, after which the substance PF1022 extracted from the cells of each strain in the supernatant was quantitatively measured

by liquid chromatography. The column used was LiChrospher 100 RP-18 (e) (Kanto Kagaku), the column temperature was 40°C, the mobile phase was 80% acetonitrile, the flow rate was 1.0 ml/min, and the substance PF1022 was detected by adsorption at 210 nm.

5 The retention time for the substance PF1022 was 5 to 6 minutes under these conditions. The experiment was repeated twice, and averages of the measurements of the substance PF1022 each extracted from the parent strain and the gene-introduced strain, into which pABP/PFsyn was introduced, are shown in Table 1.

10

Table 1

Substance PF1022 (μg/ml)	
Parent strain	88
Gene-introduced strain	222

15

The gene-introduced strain showed about 2.5 times higher productivity than the parent strain. It was revealed that the substance PF1022 productivity was enhanced by excessively expressing the cyclic depsipeptide synthetase of the present invention.

20

Further, averages of the measurements of the substance PF1022 each extracted from the parent strain and the gene-introduced strains, into which pABP/PFsynN was introduced, are shown in Table 2.

25

Table 2

Substance PF1022 (μg/ml)	
Parent strain	29
Gene-introduced strain 1	123
30 Gene-introduced strain 2	136
Gene-introduced strain 3	172

The gene-introduced strains showed 4.3 to 6.0 times higher productivity than the parent strain. It was revealed that the productivity of substance PF1022 was enhanced by excessively expressing the cyclic depsipeptide synthetase of the present invention.

## CLAIMS

1. A protein comprising an amino acid sequence selected from the group consisting of the following sequences:

- (a) an amino acid sequence of SEQ ID NO: 2, and
- (b) a modified amino acid sequence of the amino acid sequence of SEQ ID NO: 2 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and has cyclic depsipeptide synthetase activity.

2. A polynucleotide encoding the protein of claim 1.

3. A polynucleotide according to claim 2, which comprises the DNA sequence of SEQ ID NO: 1.

4. A polynucleotide selected from the group consisting of the following sequences:

- (c) a DNA sequence of SEQ ID NO: 1,
- (d) a nucleotide sequence that has at least 70% homology to the DNA sequence of SEQ ID NO: 1 and encodes a protein having cyclic depsipeptide synthetase activity,
- (e) a modified DNA sequence of the DNA sequence of SEQ ID NO: 1 that has one or more modifications selected from a substitution, a deletion, an addition and an insertion and encodes a protein having cyclic depsipeptide synthetase activity, and
- (f) a nucleotide sequence that hybridizes with the DNA sequence of SEQ ID NO: 1 under stringent conditions and encodes a protein having cyclic depsipeptide synthetase activity.

5. The polynucleotide according to claim 4, wherein sequence (d) is a nucleotide sequence that has at least 80% homology to the DNA sequence of SEQ ID NO: 1.

6. The polynucleotide according to claim 4, wherein sequence (d) is a nucleotide sequence that has at least 90% homology to the DNA sequence of SEQ ID NO: 1.

7. A recombinant vector comprising the polynucleotide of any one of claims 2 to 6.
8. A host comprising the expression vector of claim 7.
9. The host according to claim 8, which expresses a cyclic depsipeptide synthetase.
10. The host according to claim 8 or 9, which is a substance PF1022-producing microorganism.
11. A method for producing a cyclic depsipeptide, which comprises the steps of culturing the host of claim 8, 9 or 10 and collecting the cyclic depsipeptide from the culture medium.
12. The method according to claim 11, wherein the cyclic depsipeptide is the substance PF1022 and a derivative thereof.
13. A method for producing a cyclic depsipeptide synthetase , which comprises the steps of culturing the host of claim 8, 9 or 10 and collecting the cyclic depsipeptide synthetase from the culture medium.
14. The method according to claim 13, wherein the cyclic depsipeptide is the substance PF1022 and a derivative thereof.

**ABSTRACT**

The present invention provides an enzyme that synthesizes a cyclic depsipeptide, particularly the substance 1022, and a gene thereof.

5 A cyclic depsipeptide synthetase according to the present invention comprises (a) an amino acid sequence of SEQ ID NO: 2 or (b) a modified amino acid sequence of the amino acid sequence of SEQ ID NO: 2 that have one or more modifications selected from a substitution, a deletion, an addition and an insertion and has

10 cyclic depsipeptide synthetase activity. A cyclic depsipeptide synthetase gene according to the present invention comprises a nucleotide sequence encoding a cyclic depsipeptide synthetase. The present invention also provides a recombinant vector and a transformant for expressing the cyclic depsipeptide synthetase,

15 and a mass production system for the cyclic depsipeptide. The present invention further provides a method for producing the cyclic depsipeptide synthetase.

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A1

(54) Title: CYCLIC DEPSIPEPTIDE SYNTHASES, GENES THEREOF AND MASS PRODUCTION SYSTEM OF CYCLIC DEPSIPEPTIDE

WO 01/18179

(54) 発明の名称: 環状デプシペプチド合成酵素およびその遺伝子並びに環状デプシペプチドの大生産系

(57) Abstract: Enzymes synthesizing cyclic depsipeptides (in particular a substance PF1022) and genes thereof. These cyclic depsipeptide synthases contain: (a) the amino acid sequence of SEQ ID NO:2; or (b) an amino acid sequence derived from the above-described amino acid sequence by one or more modifications selected from among substitution, deletion, addition and insertion and having a cyclic depsipeptide synthase activity. The cyclic depsipeptide synthase genes comprise nucleotide sequences encoding the above-described cyclic depsipeptide synthases. Moreover, a mass production system of a cyclic depsipeptide, a process for producing a cyclic depsipeptide synthase, etc. are provided.

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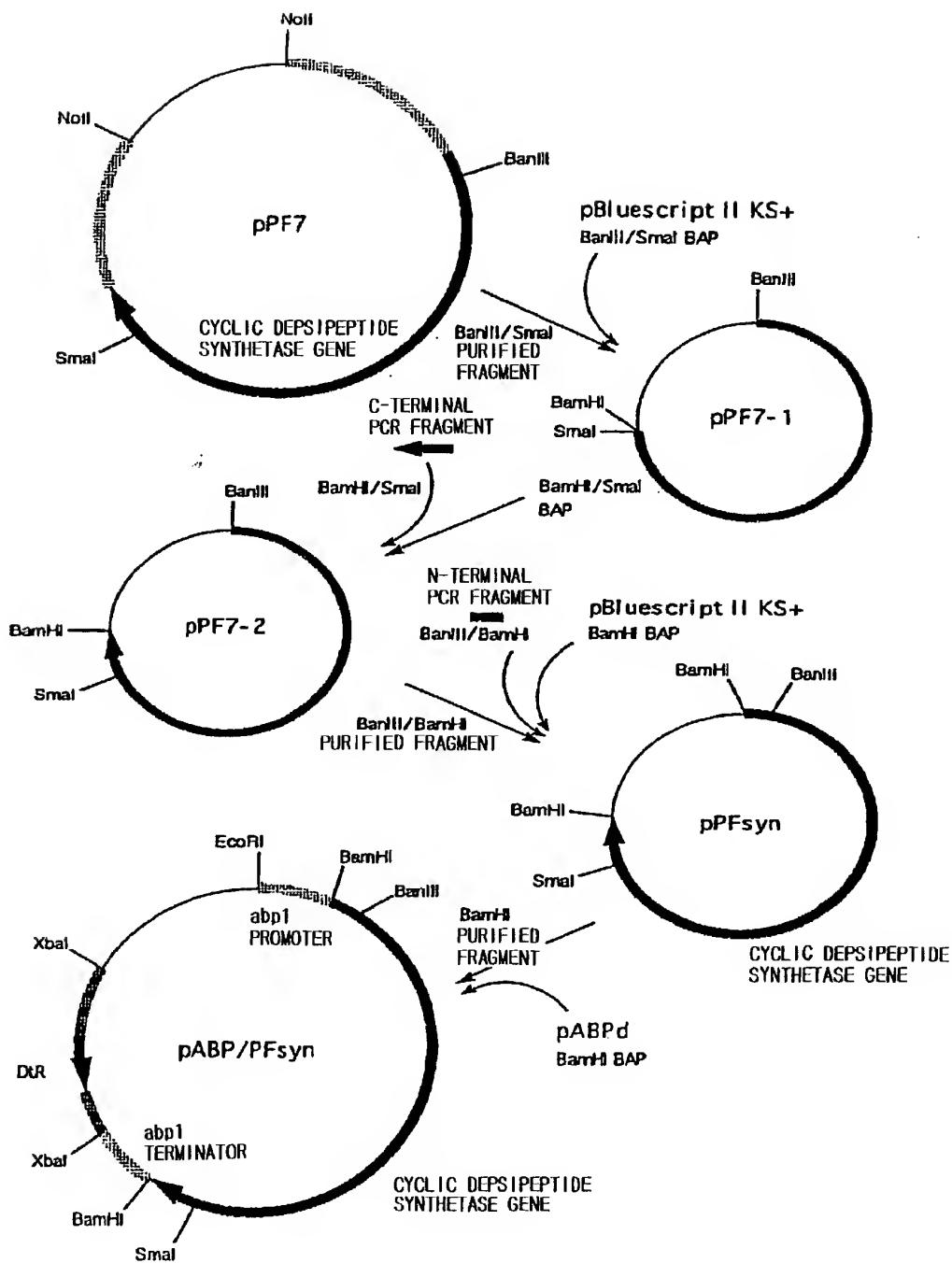


FIG. I

ABCDPQRST 107070587

214

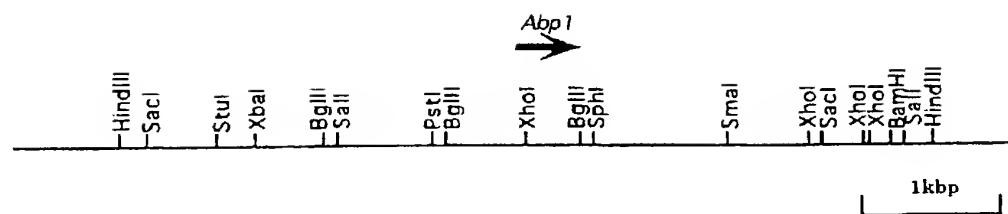
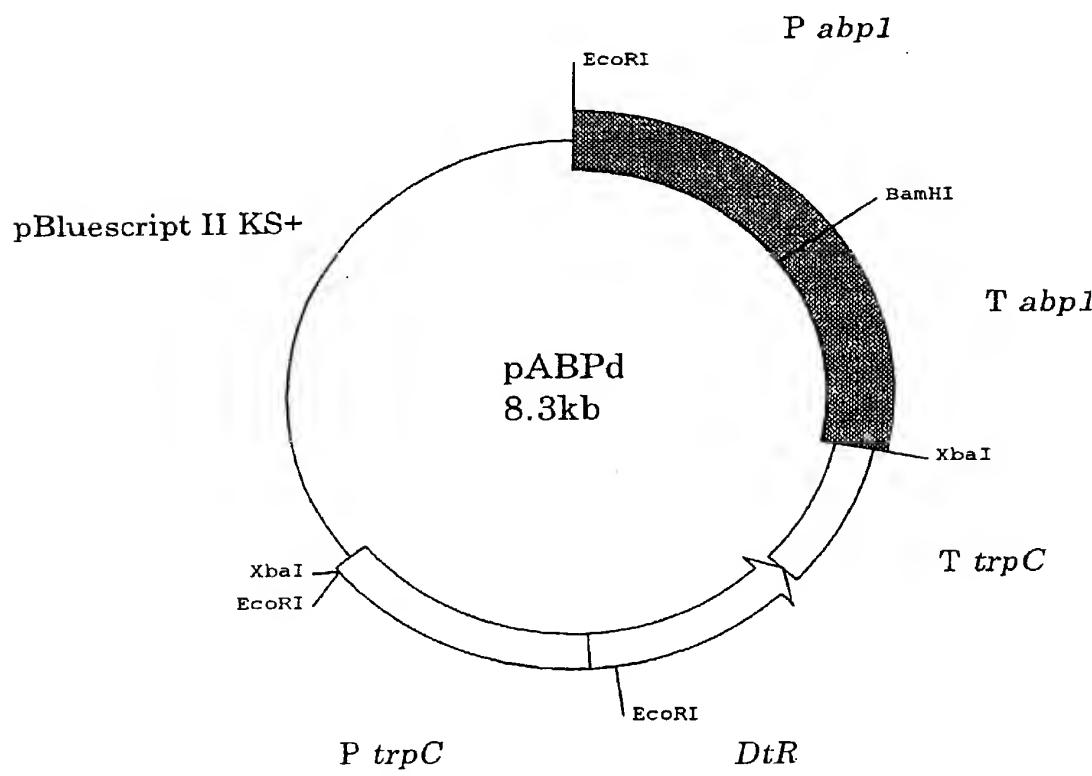
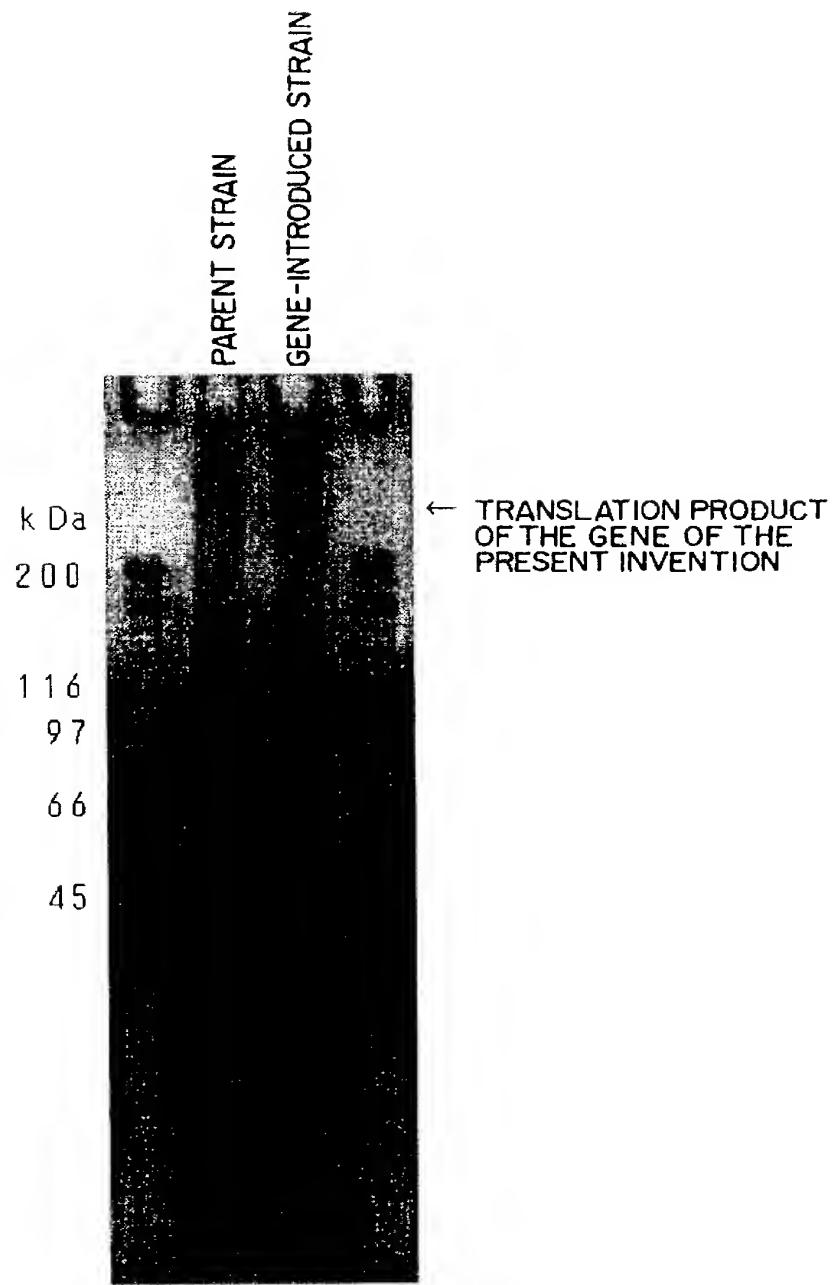


FIG. 2



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F I G. 4

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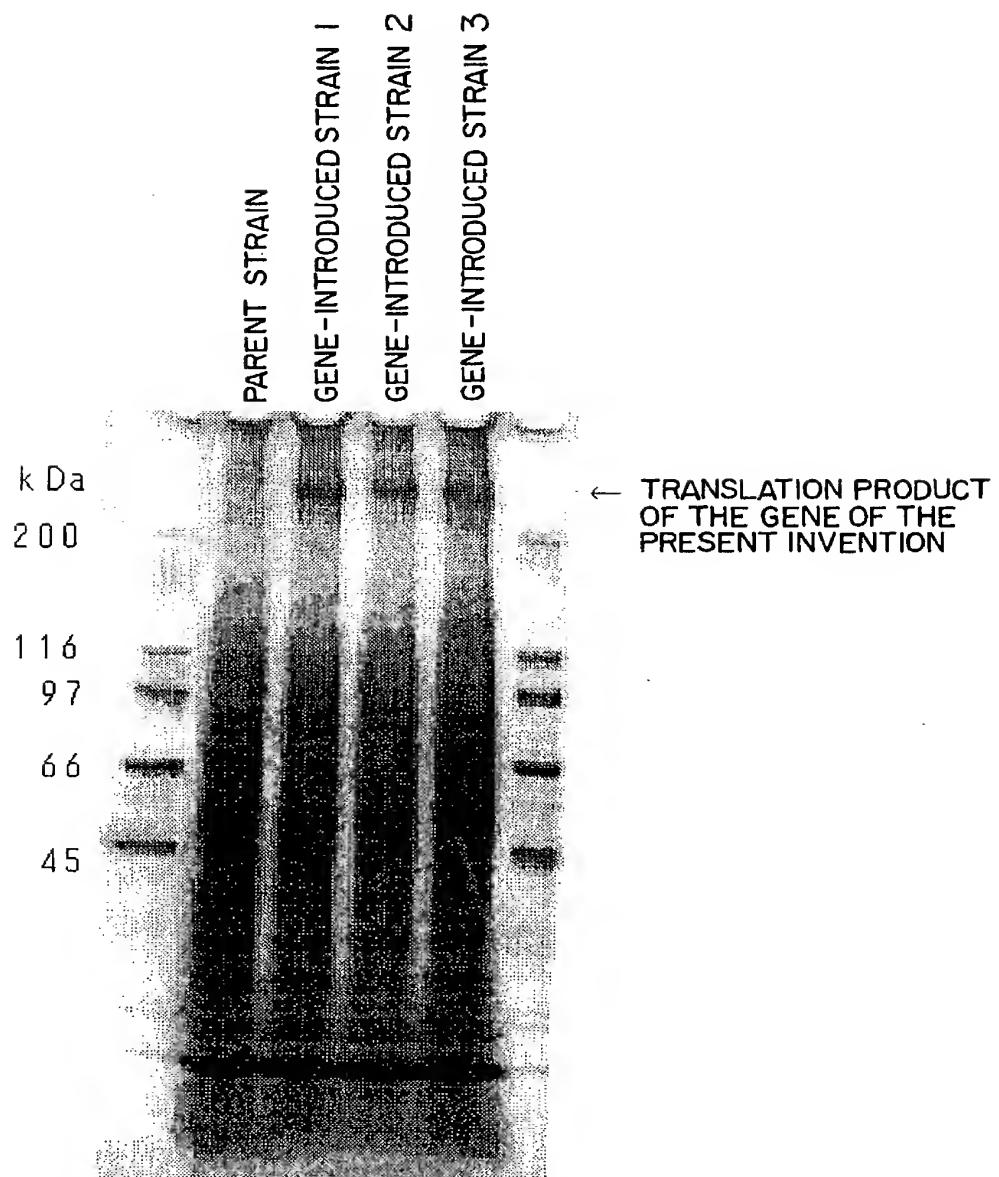


FIG. 5